Exhibit E3

HLA-B*15 subtypes in the population of north-eastern Thailand

A. Romphruk,*† K. Phongaen,‡ J. Chotechai,‡ C. Puapairoj,§ C. Leelayuwat,*† & A. V. Romphruk†§

Summary

The HLA-B*15 group is the most polymorphic HLA-B allele and so has several subtypes. These subtypes have not been defined in the population of north-eastern Thailand (NET). In a previous study, using polymerase chain reaction–sequence-specific primers (PCR-SSP), subtypes were categorized into four groups, namely: group I: HLA-B*15 (01, 04–07, 12, 14, 19, 20, 24, 25, 26N, 27, 32, 33, 34 and 35); group II: HLA-B*15 (02, 08, 11, 15, 28 and 30); group III: HLA-B*1503/4802; group IV: HLA-B*1521. Groups I and II occurred frequently (allele frequency = 8.0 and 2.5%), and thus we optimized the polymerase chain reaction–single-stranded conformation polymorphism (PCR-SSCP) method to identify HLA-B*15 subtypes of groups I and II. Eighty samples of DNA carrying HLA-B*15 from 300 healthy unrelated individuals were tested. B*1502 (52.5%) and B*1525 (13.8%) were the most common subtypes found in NET. They also showed strong linkage disequilibrium with HLA-Cw and heterogeneity of HLA-A, DR, DQ haplotypes. Although limited conclusions can be drawn from this study because of the small number of DNA references used, the baseline data will be useful in the selection of common HLA-B*15 alleles when subtyping for unrelated donor transplantations.

Introduction

Human leukocyte antigen (HLA) B15 represents a large, genetically diverse group of HLA-B alleles. It was first described and its heterogeneity recognized at the Fourth International Histocompatibility Workshop in 1970 (4th IHW) (Albert *et al.*, 1970; Thorsby & Kissmeyer-Nielsen, 1970; Thorsby *et al.*, 1970). At the 9th IHW, the heterogeneity of HLA-B15 was summarized and discussed by Chandanayingyong *et al.* (1984) and Cambon-Thomsen *et al.* (1984). At the 10th IHW, seven serological subtypes of B15 were identified and five were recognized by the World Health Organization (WHO) Nomenclature Committee as distinct specificities: B62, B63, B75, B76 and B77 (Albert *et al.*, 1989). Many new HLA-B15 related alleles have since been discovered in different ethnic groups throughout the world, especially among Southeast Asians (Alonso *et al.*, 1983; Cambon-Thomsen *et al.*, 1984; Albert *et al.*, 1989; Parham *et al.*, 1989; Pohla *et al.*, 1989; Nisperos *et al.*, 1991; Choo *et al.*, 1991, 1993; Little & Parham, 1991; Belich *et al.*, 1992; Santamaria *et al.*, 1993; Hildebrand *et al.*, 1994; Wang *et al.*, 1997). The correlations of HLA-B15 molecular structures with serological specificities and antigenic properties have been discussed (Pohla *et al.*, 1989; Hildebrand *et al.*, 1994), but the serological assignments of B*15 alleles to B62, B63, B75, B76 and B77 are complicated by cross-reactivities among the antigens encoded by these alleles (Hildebrand *et al.*, 1994; Lin *et al.*, 1996; Steiner *et al.*, 1997; Marsh *et al.*, 2001). Although the heterogeneity of HLA-B15 antigens has been described in both serological and molecular analyses, the serological complexities of this group and the corresponding molecular structures are still unclear.

In the Thai population, HLA-B15 antigens have been investigated in distinct ethnic groups such as the population of central Thailand (Bangkok) (Chandanayingyong *et al.*, 1997), Dai Lue (Chandanayingyong *et al.*, 1997), the Thai and Thai-Chinese population (Imanishi *et al.*, 1991), the population of northern Thailand (Fongsatikul *et al.*, 1997) and that of southern Thailand (Chiewsilp *et al.*, 1997). The most common serologically defined antigens were B75 and B62.

We have studied the distribution of HLA-B*15 alleles in 300 samples from the population of north-eastern Thailand (NET) based on the 12th IHW PCR-amplification refractory mutation system (ARMS). Six groups of B*15 could be defined by this protocol. Four groups of B*15 were identified in this population: B*15 (01, 04–07, 12, 14, 19, 20, 24, 25, 26N, 27 and 32–35) [allele frequency (AF) = 3.67%], B*15 (02, 08, 11, 15, 28 and 30) (AF = 8.67%), B*1503/4802 (AF = 0.5%), and B*1521 (AF = 0.5%) (Romphruk *et al.*, manuscript in preparation). Each group was carried by different HLA class I and class II haplotypes. In spite of these differences, the biological significance of the genetic heterogeneity of HLA-B15

* Department of Clinical Immunology, Faculty of Associated Medical Sciences, † Center for Research and Development of Medical Diagnostic Laboratories, ‡ Faculty of Associated Medical Sciences, and § Blood Transfusion Center, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand.

Received 26 June 2002; revised 27 August 2002; accepted 11 November 2002

Correspondence: Amornrat V. Romphruk, Blood Transfusion Center, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand. Tel./Fax: 66-43-246942; E-mail: arunrant@kku.ac.th

requires to be elucidated. Before this information can be collated, the subtypes of HLA-B15 need to be identified. Thus, our aim was to identify HLA-B*15 subtypes in NET by PCR–single-stranded conformation polymorphism (PCR-SSCP).

Materials and methods

Samples

A total of 80 HLA-B15 positive samples were obtained from 300 unrelated healthy individuals from north-eastern Thailand (Romphruk *et al.*, manuscript in preparation). All of these samples were typed for HLA-B*15 alleles using the 12th IHW PCR-ARMS protocol. Four groups were differentiated, but only two were studied by PCR-SSCP, namely: group I (n = 22), B*15 (01, 04–07, 12, 14, 19, 20, 24, 25, 26N, 27 and 32–35) and group II (n = 52), B*15 (02, 08, 11, 15, 28 and 30). The DNA references for B*15 alleles (namely B*1501, 1502, 1503, 1505, 1507, 1508, 1510, 1511, 1512, 1513, 1516, 1518, 1520, 1524, 1525, 1527, 1528 and 1532) were provided by the Japanese Red Cross Central Blood Center, Tokyo, Japan and the Australian Red Cross Blood Service, Melbourne, Australia.

Primers

A two-step PCR was performed (Fig. 1) using the same nucleotide primer sequence as Lin *et al.* (1996). In the first step, the HLA-B*15 gene fragment was amplified from genomic DNA by PCR using the group-specific primers BEX2-1 and BCT. The amplified fragment was about 742 bp in length encompassing exon 2, intron 2 and most of exon 3. A second PCR was then performed separately to amplify exon 2 (215 bp) and exon 3 (207 bp) using primer pairs BEX2-1/BEX2-FC and BEX3-1/BCT, respectively.

PCR amplifications

PCR amplifications were performed in a 10-µl reaction mixture. In the first PCR, the mixture consisted of 100 ng of genomic DNA as template, 0.5 µM of each primer, 200 µM of each dNTP, 50 mM Tris-HCl (pH 8.8), 10 mM $(NH_4)_2SO_4$, 1.0 mM $MgCl_2$ and 0.5 units of Taq DNA polymerase (Promega, Madison, WI or MHC Research Group, Khon Kaen University (Leelayuwat *et al.*, 1997)). After preheating at 94 °C for 2 min, 35 PCR cycles were performed, followed by final extension at 72 °C for 10 min. Each amplification cycle consisted of 1 min each of denaturing (94 °C), annealing (54 °C) and extension (72 °C). The PCR was carried out in a Gene Amp PCR system 9600 (Perkin Elmer Corporation, Norwalk, CT).

The reaction mixture for the second PCR was the same as that for the first PCR, except that we used 1 µl of the first PCR product as the template, 0.8 mM $MgCl_2$ for exon 2 and 1.2 mM $MgCl_2$ for exon 3. The amplification was performed for 20 cycles, after preheating to 94 °C for 2 min and final extension of 10 min at 72 °C. Each amplification cycle consisted of denaturing (94 °C) for 30 s, annealing (58 °C) for 30 s, and extension (72 °C) for 1 min. The presence or absence of PCR product was determined by gel electrophoresis. Five microlitres of PCR product was run on a 1% agarose gel containing 0.5 µg ml^{-1} of ethidium bromide and visualized under UV illumination.

SSCP fragment analysis

The exon 2 and 3 fragments amplified by the second PCR were analysed separately using the SSCP method (Lin *et al.*, 1996; Maruya *et al.*, 1996). Electrophoresis was carried out in a Miniprotein II polyacrylamide gel (Mini-PROTEAN II, Biorad, Hercules, CA) (Table 1). The single-stranded DNA fragments separated in the gel were detected by silver staining.

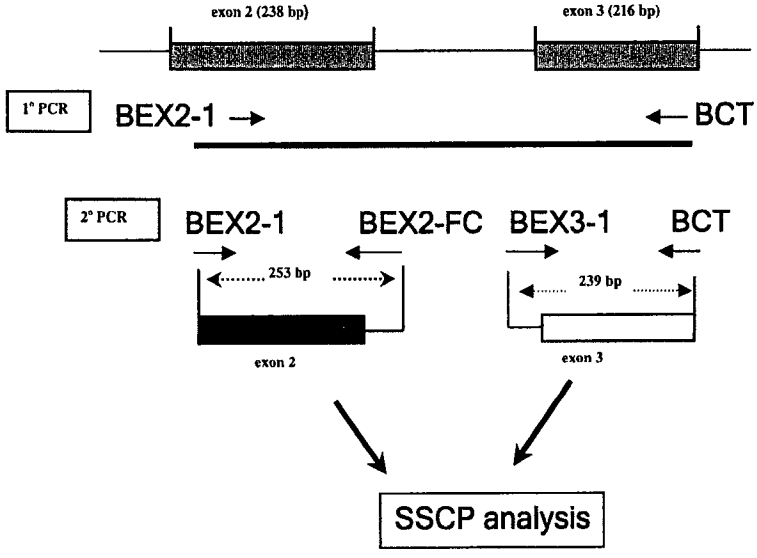

Figure 1. The strategy of SSCP fragment analysis of exons 2 and 3 in HLA-B*15 alleles. Primers BEX2-1 and BCT are group-specific primers. BEX2-1/BEX2-FC and BEX3-1/BCT amplified the exon 2 and exon 3 seperately.

Table 1. SSCP conditions for groups I and II of the B*15 alleles

Group		Electrophoretic temperature (°C)	Voltage (v)	Time (min)	Gel concentration	Acrylamide:bis
Group I	exon 2	4	200	150	10%	39 : 1
B15* (01, 04–07, 12, 14, 19, 20, 25, 26N, 27, 32–35)	exon 3	4	150	150	7%	50 : 1
Group II	exon 2	4	150	150	10%	50 : 1
B*15 (02, 08, 11, 15, 28, 30)	exon 3	4	150	60	7%	50 : 1

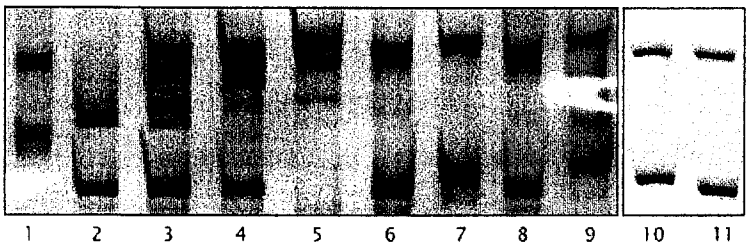

Figure 2. PCR-SSCP analysis of HLA-B15 group I (1501, 1505, 1507, 1512, 1520, 1524, 1525, 1527, 1532). Lanes 1 (B*1525), 2 (B*1532), 3 (B*1507), 4 (B* 1520), 5 (B* 1512), 6 (B* 1524), 7 (B*1527), 8 (B* 1501) and 9 (B* 1505) were distinguished using exon 3 fragment analysis. Lanes 10 (B*1501) and 11 (B*1524) by exon 2.

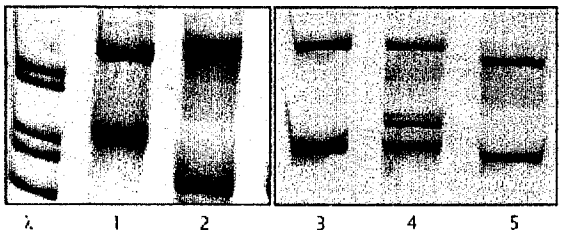

Figure 3. PCR-SSCP analysis of HLA-B15 Group II (1502, 1508, 1511, 1528). Lanes 1 (B*1502) and 2 (B*1508/1511/1528) were done using exon 3 fragments analysis. Lanes 3 (B*1508), 4(B*1511) and 5 (B*1528) by exon 2.

Reproducibility of the test

To ensure reproducibility, the DNA references were tested within and between runs for both PCR amplification and SSCP fragment analysis.

Results

SSCP fragment analysis

Most of alleles in group I had nucleotide sequence differences in exon 3, so analysis was used to discriminate alleles B*1505, 07, 12, 20, 25, 27 and 32. The other two alleles, B*1501 and B*1524, were defined by the exon 2 SSCP analysis (Fig. 2). In group II, exon 3 SSCP analysis discriminated B*1502 from B*1508, 1511 and 1528 and exon 2 discriminated B*1508, B*1511 and B*1528 (Fig. 3).

Reproducibility of the test

The SSCP analysis of DNA references for exon 2 for both groups I and II showed identical banding patterns 'within' and 'between' runs. The exon 3 analysis showed slight differences in banding patterns on the 'between run' assay, but these alleles were easily defined. Thus, the DNA references were included in every SSCP analysis.

B*15 alleles in NET

Eight different patterns of HLA-B*15 alleles (groups I and II) were detected in NET (Table 2). The common alleles were B*1502 (52.5%), B*1525 (13.8%) and B*1528 (5.0%). Three samples of group I [B*15 (01, 07)?] could not be clearly defined because extra bands from the other HLA-B allele in these samples could not be excluded. One of the 22 samples from group I and two of the 52 in group II were undefined because they gave different banding patterns from the DNA references. They might have been B*15 (04, 06, 14, 19 or 33–35) or B*15 (15 or 30) for which we have no DNA references. Five samples from group I and three from group II could not be analysed because DNA samples were not available. The comparison of B*15 alleles in NET and other populations is presented in Table 3.

Haplotype analysis

Table 4 summarizes the possible association of HLA-B*15 alleles with other HLA alleles. The data for other loci are from a previous study (Romphruk *et al.*, manuscript in preparation). Only four haplotypes in Table 4 could be confirmed by family segregation analysis. Most HLA-B*1502 haplotypes (92.85%) carried HLA-Cw*08 but carried diverse alleles at HLA-A, -DR and -DQ. All HLA-B*1525 and B*1521 haplotypes carried HLA-Cw*04 but carried diverse alleles at HLA-A, -DR and -DQ. In addition, associations of B*1528 and B*1532 with Cw*07 (01–03) were observed.

Table 2. B*15 alleles in HLA-B15 positive samples (n = 80) from 300 healthy individuals from north-eastern Thailand

B*15[a]	B*15 alleles	n (%) (n = 80)
Group I	1501	—
	1505	—
	1507	—
	1512	—
	1520	—
	1524	—
	1525	11 (13.8%)
	1527	—
	1532	2 (2.5%)
	15 (01, 07)?	3 (3.7%)
	15 (04, 06, 14, 19, 33, 34, 35)	1 (1.3%)
	Not done	5 (6.2%)
Group II	1502	42 (52.5%)
	1508	—
	1511	1 (1.3%)
	1528	4 (5.0%)
	15 (15, 30)?	2 (2.5%)
	Not done	3 (3.7%)
Group III	1503/4802	3 (3.7%)
Group IV	1521	3 (3.7%)
Group V	15 (13, 16, 17)	0
Group VI	15 (09, 10, 18, 23)	0

[a] The B15 groups were defined according to the 12th IHW PCR-ARMS.

Table 3. Percentage of HLA-B*15 subtypes in various populations

B*15	NET (n = 80)	Vietnamese[e] (n = 43)	Caucasian[e] (n = 70)	Ni-Nanuatu[e] (n = 37)	Bubi[e] (n = 30)	Koreans[f] (n = 237)	Japanese[g] (n = 53)
1501	3.75[a]	20.9	85.7[d]	5.4		35.0[d]	30.2[d]
1502	52.5	55.8				1.5[d]	0.9[d]
1503	3.7[b]				53.3[d]		ND
1506	ND			89.19[d]			ND
1507			1.4			4.6	4.7
1509			1.4				ND
1510					40.0[d]		ND
1511	1.3					7.6[d]	2.8
1512		14.0[d]					ND
1515	2.5[c]					ND	ND
1516					10.0		ND
1517			5.7			0.002	ND
1518	ND		7.1			3.0	10.4
1521	3.7	2.3		8.1			ND
1525	25.6	14.0				0.002[d]	ND
1527		2.3				1.5	0.9
1528	5.0						ND
1532	2.5					ND	ND
1538	ND	ND	ND	ND	ND	0.004	ND

Blank = not found. ND = not done/no data. [a] B15 (01 or 07); [b] B1503/4802; [c] B15 (15 or 30); [d] significant difference compared to NET (corrected $P < 0.05$); [e] data from Barnardo *et al.* (1998); [f] data from Lee *et al.* (2000); [g] data from Saito *et al.* (2000).

Discussion

HLA-B*15 is a diverse group of alleles of the HLA-B locus and is found throughout the world, suggesting that its lineage is as old as *Homo sapiens* (Hildebrand *et al.*, 1994). By DNA sequencing of exons 2 and 3, 68 B*15 alleles were defined (Marsh *et al.*, 2001). B*15 alleles associated with specific ethnic groups have been described, including B*1518 in Asian Indians and B*1521 in Australian Aborigines (Lienert *et al.*, 1995).

We used PCR-SSCP to define the B*15 allele in NET. This technique depends on the conformation of single-stranded DNA to produce the different banding patterns, so DNA references are needed for comparisons. However, the references themselves varied under different conditions. Unfortunately, we did not have all the DNA references,

Table 4. Possible associations of B*15 alleles with other HLA loci

B*	A	Cw*	DRB1*	DQB1*	%
1502		08			92.85 (39/42)
	11	08	1202	0301	26.19 (11/42)
	24	08	1501	0601	26.19 (11/42)
	11	08	1502	0501	4.76 (2/42)
1525		04			100 (11/11)
		04	1106	0301	27.27 (3/11)
		04	1502	0501	18.18 (2/11)
		04	1502	0502	18.18 (2/11)
1521	34/66	04			100 (3/3)
1528		07(01–03)			50 (2/4)
1532		07(01–03)			100 (2/2)

so only nine of the 17 alleles for group I [B*15 (01, 05, 07, 12, 20, 24, 25, 27 and 32)] and four of the six for group II [B*15 (02, 08, 11 and 28)] were included in our study. Specific primers can amplify other alleles at the B locus, such as B*4601, B*5801, B*38, B*39 and B*35 (Lin *et al.*, 1996), so, in addition to the two alleles per locus in individual samples, the SSCP pattern revealed other bands which were not from B*15 alleles. Thus, including other known alleles present in the sample in the SSCP fragment analysis will help to clarify the B*15 bands.

HLA-B15 allelic types and their frequencies have previously been shown to vary in different populations (Marsh *et al.*, 2001; Lee *et al.*, 2000). Frequencies of some of the most common B*15 alleles have been determined: B*1502 in NET (52.5%) and Vietnamese (55.8%); B*1501 in Caucasians (85.7%), Koreans (35.0%), and Japanese (30.2%); B1506 in Ni-Vanuatu (89.2%); B*1503 and B*1510 in Bubi (53.3 and 40.0%). The distribution of the B*15 alleles in NET is similar to that in the Vietnamese, except for B*1512. Compared to other Thai groups (Thais and Thais-Chinese, Thais from Central Thailand and Dai Lue), B*75 (encoded by B*1502, 1508, 1511 and 1521), B62 (encoded by B*1501, 1504–1507, 1515, 1520, 1524, 1525, 1527, 1530, 1532, 1545, and 1548) and B77 (encoded by B*1513) were found more frequently (Chandanayingyong *et al.*, 1997; Imanishi *et al.*, 1991). It is possible that B75 was encoded by B*1502 and that most of B62 was encoded by B*1525 in NET. In contrast, B*1501 was the predominant allele for the B62 antigen in Koreans (166 in 198 samples) but B*1525 was rare (1 in 198 samples) (Lee *et al.*, 2000). B*1513 was not found in the present study, indicating that this allele is rare in north-eastern Thailand. In a separate study, we found this allele in only one in 240 cases of cholangiocarcinoma in patients from north-eastern Thailand (Romphruk *et al.*, manuscript in preparation). This confirms our observation that the distribution of MHC alleles in the population of north-eastern Thailand is different from that in the population of Central Thailand, which shows a high degree of Thai-Chinese admixture (Romphruk *et al.*, 1999).

HLA-B15, combined with B4601, is the most common (22–30%) HLA-B allele in south-east Asians, including the Chinese. Hildebrand *et al.* (1994) and Lin *et al.* (1996) proposed a phylogenetic tree for 26 HLA-B15 alleles and a network of structure relationships among these alleles. B4601 may have evolved from B*1501 — the putative original B*15 allele (Zemmour *et al.*, 1992; Hildebrand *et al.*, 1994; Lin *et al.*, 1996;). B*4601 and B*1501 differ in the short codon position 66–76, which may be the result of segmental conversion between B*1501 and Cw*01. Thus, HLA-B*4601 in Asians may have originated from B*1501 via gene conversion. This hypothesis, combined with selective pressure, may explain the low frequency of B*1501 and the high frequency of B*4601 in these populations.

The possible associations of B*15 with other loci are presented in Table 4. The possible B*15–Cw* association showed several different patterns. B*1502 was strongly associated with Cw*08, B*1525 with Cw*04, and B*1528 and B*1532 with Cw*07 (01–03). B*1502 and B*1525 were more diverse in their associations with HLA-A, Cw, DR and DQ than other B*15 alleles. This suggests that these two alleles may be relatively old alleles in the Thai population, similar to B*1501 and B*1518 in Koreans. The diversity of haplotypes is likely to be the result of recombination between HLA-B and other loci during the long period of haplotype formation in the population (Lee *et al.*, 2000). The strong linkage disequilibrium between B*15 alleles and HLA-Cw will be useful in discriminating the B15 subgroups, especially where B*15 subtyping is not available.

In conclusion, we defined B*15 alleles and the heterogeneity of haplotypes carrying HLA-B15 in NET. High-resolution typing will be necessary to select unrelated donors for organ and bone marrow transplantation. The data obtained in this and similar studies will facilitate the selection of common B*15 alleles for subtyping in these populations.

Acknowledgements

We thank Professor Katsushi Tokunaga and Dr Yoshihide Ishikawa of the Japanese Red Cross Central Blood Center, Tokyo, Japan and Mrs Rhonda Holdsworth of the Australian Red Cross Blood Service, Melbourne, Australia. Dr Chanvit Leelayuwat is a Thailand Research Fund Scholar supported by the Thailand Research Fund (TRF). The authors thank Mr Bryan Roderick Hamman for assistance with the English and the presentation of the manuscript.

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